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Diffusion of Rifampin and Vancomycin through a *Staphylococcus epidermidis* Biofilm

W. MICHAEL DUNNE, JR.,[1,2]†* EDWARD O. MASON, JR.,[2] AND SHELDON L. KAPLAN[2]

Departments of Pathology[1] and Pediatrics,[2] Baylor College of Medicine and Texan Children's Hospital, Houston, Texas 77030-2399

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Using an equilibrium dialysis chamber, we evaluated the penetration of vancomycin, rifampin, or both through a staphylococcal biofilm to simulate treatment of an infected biomedical implant. A biofilm of ATCC 35984 (slime-positive *Staphylococcus epidermidis*; vancomycin MIC and MBC, 1 and 2 μg/ml, respectively; rifampin MIC and MBC, 0.00003 and 0.00025 μg/ml, respectively) was established on the inner aspect of the dialysis membrane (molecular mass exclusion, 6,000 kDa). Serum containing vancomycin (40 μg/ml), rifampin (20 μg/ml), or a combination of both was introduced into the inner chamber of the dialysis unit (in direct contact with the biofilm), and serum alone was added to the outer chamber. Rifampin and vancomycin concentrations in both chambers were determined over a 72-h period. In the absence of rifampin, the concentration of vancomycin in the outer chamber exceeded the MBC for the organism after 24 h, and the MBC increased to nearly 8.0 μg/ml by 72 h, demonstrating that therapeutic levels of vancomycin can penetrate a staphylococcal biofilm. However, viable bacteria were recovered from the biofilm after 72 h of treatment with no apparent increase in the MIC or MBC of vancomycin. Similarly, concentrations of rifampin exceeding the MBC were detected in the outer chamber after 24 h of treatment, but viable organisms were recovered from the biofilm after 72 h of treatment. In this case, the rifampin MBCs for surviving organisms increased from 0.00025 to >128 μg/ml. The combination of agents prevented the development of resistance to rifampin, improved the perfusion of vancomycin through the biofilm, and decreased the penetration of rifampin but did not sterilize the membrane. These observations provide evidence that bactericidal levels of vancomycin, rifampin, or both can be attained at the surface of an infected implant. Despite this, sterilization of the biofilm was not accomplished after 72 h of treatment.

Infection is a frequent complication associated with the use of transcutaneous and implantable biomedical devices such as intravascular and peritoneal catheters, cerebrospinal fluid shunts, and prosthetic heart valves (2, 6, 19, 21). The infecting microorganism is often a member of the normal mucocutaneous microflora including *Candida* and *Corynebacterium* species, but coagulase-negative staphylococci (CONS), especially *Staphylococcus epidermidis*, predominate as agents of biomedical implant infections (8).

The pathogenesis of implant infection begins with primary bacterial adhesion to the exposed surface of the device. For CONS, primary adhesion is thought to be mediated through hydrophobic interactions between the cell wall and the surface of the implant and/or ligand-specific binding to host-derived factors which coat the implant (9, 15, 18). Regardless of the process, the surface of the infected device is eventually enveloped in a matrix of bacteria, extracellular bacterial products, and exogenous host factors collectively referred to as a biofilm layer (8). Once a biofilm has been established, therapeutic options are often limited to long-term antimicrobial therapy, removal of the device, or both. Antimicrobial therapy alone, even with agents demonstrating in vitro efficacy, is seldom effective for sterilization of CONS-infected implants (4, 21).

At least two scenarios could explain the refractivity of biomedical implants infected with CONS to antimicrobial therapy. (i) The thickness and chemical composition of the biofilm prevent the perfusion of antimicrobial agents so that inhibitory or bactericidal levels are not attained at the surface of the device, or (ii) the microenvironment of the biofilm or the metabolic state of the bacteria within the biofilm negates the antimicrobial activities of agents with in vitro efficacy. To address the former, we developed an in vitro model of *S. epidermidis* implant infection using an equilibrium dialysis chamber that allows measurement of the diffusion of antimicrobial agents through the bacterial biofilm layer. For the study, we examined the diffusion of vancomycin, rifampin, or a combination of both through an established *S. epidermidis* biofilm.

MATERIALS AND METHODS

Bacterial strain and antimicrobial agents. All experiments were conducted with *S. epidermidis* ATCC 35984, a slime-producing strain previously characterized by Christensen et al. (3). By broth macrodilution testing with Mueller-Hinton broth (12), the organism was shown to be susceptible to vancomycin (MIC, 1 μg/ml; MBC, 2 μg/ml) and rifampin (MIC, 0.00003 μg/ml; MBC, 0.00025 μg/ml). Synergy studies done by checkerboard titration showed that the combination of agents reduced the rifampin MIC to 0.000008 μg/ml and the vancomycin MIC to 0.5 μg/ml, for a calculated fractional inhibitory concentration of 0.77, indicating no synergistic activity against the test strain (17). Standard preparations of vancomycin and rifampin were obtained from Eli Lilly (Indianapolis, Ind.) and Marion Merrell Dow, Inc. (Cincinnati, Ohio), respectively. Stock solutions of rifampin were solubilized in dimethyl sulfoxide (Sigma, St. Louis, Mo.) and were diluted to working concentrations in fetal calf serum.

* Corresponding author.
† Present address: Department of Pathology, Henry Ford Medical Center, 2799 West Grand Boulevard, Detroit, MI 48202-2689.

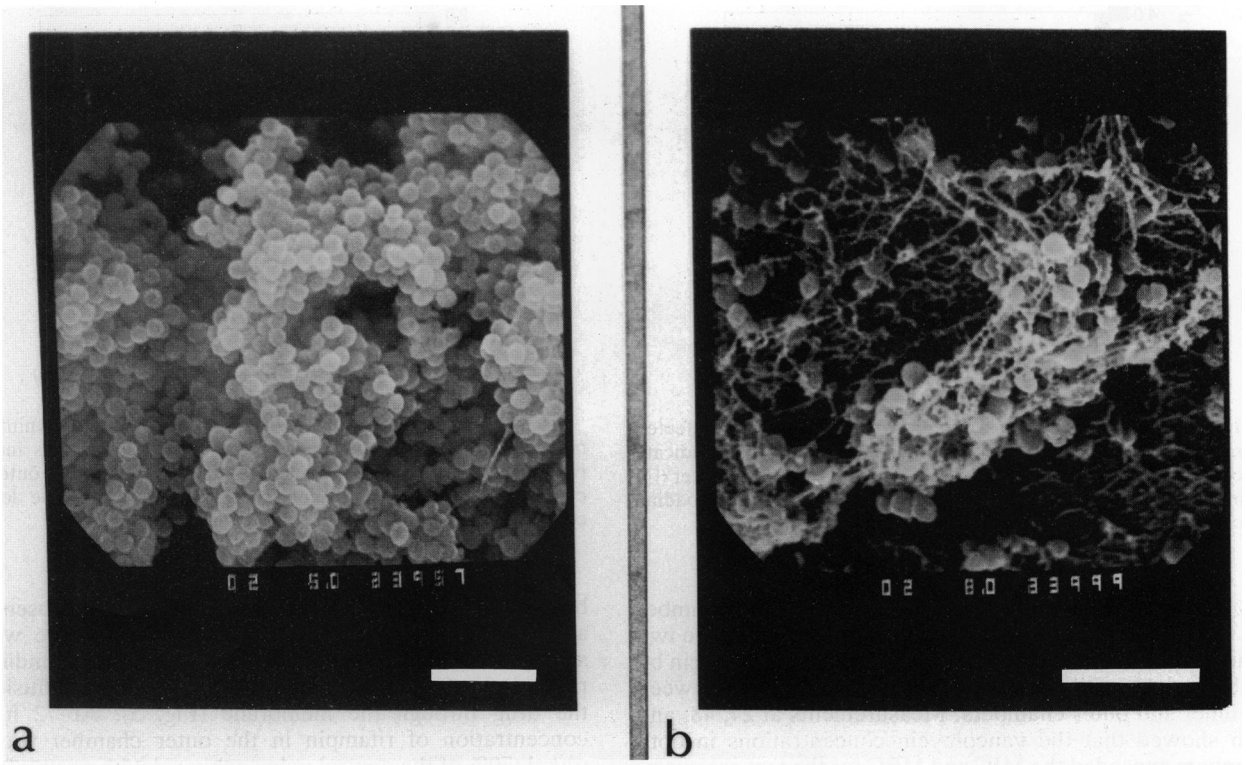

FIG. 1. Scanning electron micrograph of an untreated biofilm of *S. epidermidis* formed on the surface of an equilibrium dialysis membrane (a) and the same biofilm after 72 h of contact with vancomycin and rifampin in concentrations that exceeded the MIC and MBC for the organism (b). Despite marked differences in the appearance of the biofilm, we recovered from the latter viable organisms for which MICs of either agent were unaltered. The bars in the lower right of each panel are approximately 5 μm.

The final concentration of dimethyl sulfoxide was found to have no effect on the morphology of staphylococcal biofilms or on the results of susceptibility studies.

In vitro biofilm model. The model that we used to evaluate the diffusion of antimicrobial agents through a staphylococcal biofilm was similar to the system described by Hoyle et al. (10) and was constructed as follows. (i) Both sides of an ethylene oxide-sterilized equilibrium dialysis chamber (model H40262; Scienceware; Bel-Art Products, Pequannock, N.J.) separated by a 6,000-kDa-molecular-mass-cutoff dialysis membrane were aseptically filled with 10 ml of Trypticase soy broth. One side (the inner side) was inoculated with the test strain, and the chamber was incubated at 35°C with the inner side up. After 48 h of incubation, a dense, uniform staphylococcal biofilm covered the inner surface of the dialysis membrane (Fig. 1a). At that time, the growth medium was removed from both sides of the apparatus. Ten milliliters of fetal calf serum containing either vancomycin (40 μg/ml), rifampin (20 μg/ml), or both was introduced into the inner side of the chamber (i.e., to the exposed surface of the biofilm), and 10 ml of fetal calf serum without antimicrobial agents was added to the outer chamber. Samples were aseptically removed from both sides at predetermined intervals, and the concentration of vancomycin or rifampin was determined. Following a 72-h incubation period, the dialysis membrane was removed from the chamber. The biofilm was sampled for the presence of viable bacteria and the MICs and MBCs of vancomycin and rifampin for the recovered organisms were determined. In one experiment, the membrane was fixed in 3% glutaraldehyde and was processed for scanning electron microscopy. To determine the effect of the dialysis membrane on the diffusion kinetics of vancomycin or rifampin, parallel experiments were performed with uninfected membranes.

Measurement of antimicrobial agents. Vancomycin concentrations were measured by using the Abbott TDx system (Abbott Laboratories, Abbott Park, Ill.), while rifampin concentrations were analyzed spectrophotometrically at A_{478}. For the latter, the standard curve for rifampin demonstrated linearity over the concentration range used throughout the study. The assay was sensitive to at least 0.1 μg/ml (data not shown).

RESULTS

Diffusion of vancomycin. The equilibrium dialysis profile of vancomycin across an uninfected dialysis membrane is shown in Fig. 2. The diffusion pattern produced in fetal calf serum was nearly identical to that obtained with phosphate-buffered saline as a diluent, indicating little or no interference in the diffusion of vancomycin across the membrane caused by serum proteins (data not shown). Without a biofilm, the vancomycin concentration in the outer chamber reached 9 μg/ml after 24 h of incubation and 15 μg/ml by 72 h; the latter was nearly 40% of the loading concentration. The presence of a staphylococcal biofilm markedly altered the diffusion of vancomycin across the membrane (Fig. 3). At 24 h, the vancomycin concentration in the outer chamber was 2.75 μg/ml, only 30% of the concentration measured in the absence of a biofilm. After 72 h of incubation, less than

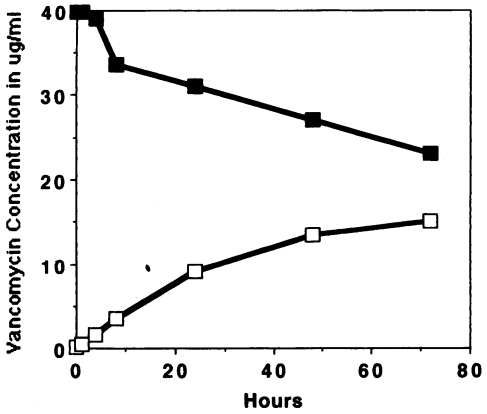

FIG. 2. Equilibrium dialysis of vancomycin across an uninfected membrane (molecular mass cutoff, 6,000 kDa). Symbols indicate vancomycin concentrations measured in the inner (■) and outer (□) chambers of the dialysis unit over a 72-h period. The loading concentration in the inner chamber was 40 μg/ml.

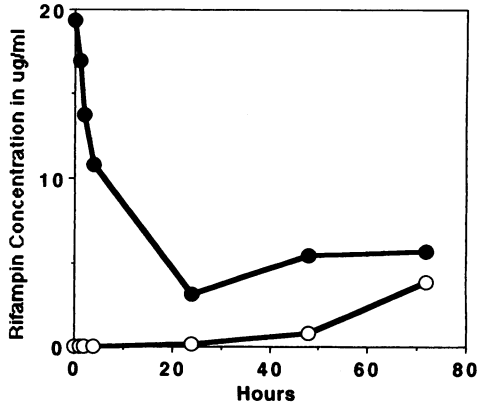

FIG. 4. Equilibrium dialysis of rifampin across an uninfected membrane (molecular mass cutoff, 6,000 kDa). Symbols indicate rifampin concentrations measured in the inner (●) and outer (○) chambers of the dialysis unit over a 72-h period. The loading concentration in the inner chamber was 20 μg/ml.

20% of the vancomycin introduced into the inner chamber had diffused across the membrane. A comparison of the two diffusion patterns indicates a rapid uptake of vancomycin by the bacterial biofilm, with a secondary equilibration between the inner and outer chambers. Measurements at 24, 48, and 72 h showed that the vancomycin concentrations in both chambers exceeded the MIC and MBC for the test organism. Despite this, viable bacteria for which there was no change in the MIC and MBC of vancomycin were recovered from the biofilm after 72 h of incubation.

Diffusion of rifampin. Unlike vancomycin, the equilibrium dialysis pattern of rifampin across an uninfected membrane showed a rapid decline in the concentration in the inner chamber, with a slow accumulation in the outer chamber, suggesting a high degree of binding to the dialysis membrane or to the inner surface of the dialysis chamber (Fig. 4). After 72 h of incubation, only 5.7 μg of rifampin per ml remained in the inner chamber, while the rifampin concentration in the outer chamber had risen to 4 μg/ml. Therefore, approximately 50% of the rifampin introduced into the inner chamber was adsorbed from the aqueous phase in the absence of a biofilm. Colonization of the dialysis membrane with a staphylococcal biofilm reduced the nonspecific binding of rifampin to the membrane but did not facilitate diffusion of the drug through the membrane (Fig. 5). At 72 h, the concentration of rifampin in the outer chamber was 3.1 μg/ml (79% of the control value achieved with an uninfected membrane), while the rifampin concentration in the inner chamber stabilized at 14.7 μg/ml. Once again, viable bacteria were recovered from the biofilm after 72 h of incubation. However, the MIC and MBC of rifampin for the recovered organisms had increased to greater than 128 μg/ml.

Diffusion of vancomycin and rifampin. The combination of vancomycin and rifampin affected the diffusion of both antimicrobial agents across the staphylococcal biofilm (Fig. 6). Although the general diffusion profile of rifampin was basically unchanged, the total accumulation of the drug in the outer chamber was reduced by 51% compared with the value obtained without the addition of vancomycin (from 3.5 to 1.6 μg/ml). Conversely, the combination of agents facili-

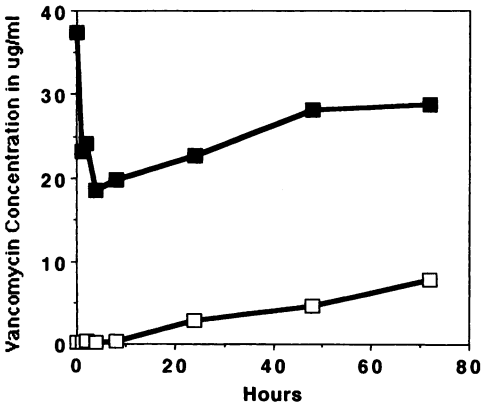

FIG. 3. Equilibrium dialysis of vancomycin across a staphylococcal biofilm. Concentrations of vancomycin were determined over a 72-h incubation period in both the inner (■) and outer (□) chambers of the dialysis unit. The inner chamber corresponds to the side of the membrane colonized by *S. epidermidis*. The loading concentration in the inner chamber was 40 μg/ml.

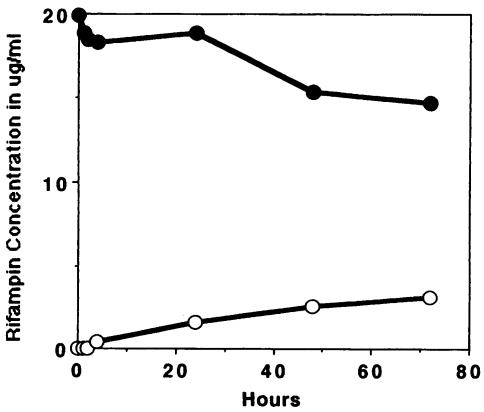

FIG. 5. Equilibrium dialysis of rifampin across a staphylococcal biofilm. Concentrations of rifampin were determined over a 72-h incubation period in both the inner (●) and outer (○) chambers of the dialysis unit. The inner chamber corresponds to the side of the membrane colonized by *S. epidermidis*. The loading concentration in the inner chamber was 20 μg/ml.

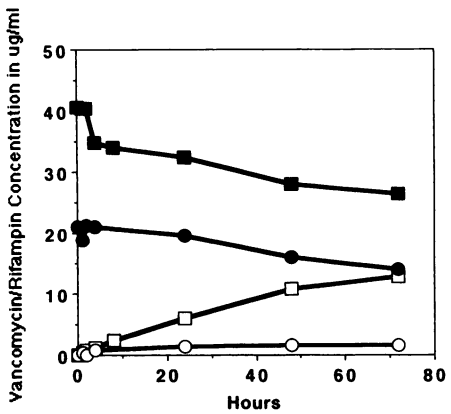

FIG. 6. Equilibrium dialysis of vancomycin and rifampin across a staphylococcal biofilm over a 72-h incubation period. Symbols indicate the concentration of vancomycin in the inner (■) and outer (□) chambers and the concentration of rifampin in the inner (●) and outer (○) chambers. Loading concentrations in the inner chamber were 40 and 20 μg/ml for vancomycin and rifampin, respectively.

tated the diffusion of vancomycin across the biofilm. At 24 and 72 h, the levels of vancomycin attained in the outer chamber were 215 and 165%, respectively, of the values obtained in the absence of rifampin. Scanning electron microscopy of the membrane after 72 h of treatment with both drugs showed a marked change in the appearance of the biofilm, with a considerable reduction in the density of adherent bacteria and a matrix of fibrous material over the surface of the membrane to which bacteria were attached (Fig. 1b). As before, viable organisms were recovered from the membrane after 72 h of incubation, even though the MICs and MBCs of vancomycin and rifampin on both sides of the biofilm were in excess of those for the organisms. Susceptibility testing of the organism recovered after 72 h of treatment showed that the combination of antimicrobial agents prevented the development of resistance to rifampin (MIC and MBC, 0.001 and 0.015 μg/ml, respectively). The MIC of vancomycin for the recovered organism also remained unchanged, but the MBC increased fourfold, from 2 to 8 μg/ml.

DISCUSSION

Despite the availability of antimicrobial agents with excellent in vitro activity, the treatment of biomedical implant infections caused by CONS remains problematic. The unusual resistance of implant infections to antimicrobial therapy is not limited to CONS but is shared by a number of bacteria including *Pseudomonas aeruginosa* and *Staphylococcus aureus* (1, 10). The apparent incongruity between in vitro susceptibility and clinical response to antimicrobial agents can be partially explained by the physiology of bacteria in a biofilm. Anwar et al. (1) have shown that the susceptibility of *S. aureus* to tobramycin and cephalexin decreases significantly with time when the organism is grown on a solid surface (silicon tubing) compared with organisms grown in suspension 1E, planktonic bacteria. The same observations have been made with *P. aeruginosa* (1, 10) as well as *S. epidermidis* (14, 16). In addition to biofilm formation, certain strains of CONS are capable of producing an extracellular polysaccharide or slime layer (2, 3). In vivo, the slime polysaccharide complexes with endogenous host factors to form a matrix which enhances the stability of the biofilm on the surface of the implant and also provides a certain degree of protection from host defenses (8, 11). It has been suggested that the mechanical barrier of the CONS matrix, which has been shown to be as thick as 140 μm (14), might limit the diffusion of antibiotics through the biofilm so that effective concentrations are not attained. In support of this hypothesis, Younger et al. (21) have provided clinical evidence that ventriculoperitoneal shunts infected with slime-producing strains of CONS are less likely to respond to appropriate antimicrobial therapy than shunts infected with slime-negative strains. Using a membrane dialysis model similar to ours, Hoyle et al. (10) have shown that complexes of Ca^{2+} and the alginic acid exopolysaccharide of *P. aeruginosa* prevent the diffusion of piperacillin through the biofilm layer. Taken together, those studies provide evidence that changes in bacterial physiology, altered environmental conditions, and physical barrier protection could provide adherent CONS with several mechanisms for eluding antimicrobial therapy. The intent of our model was to determine to what extent barrier protection contributed to the increased resistance by preventing diffusion of antimicrobial agents to the innermost layer of the bacterial biofilm nearest the surface of the infected implant.

Using this model and clinically achievable levels of rifampin and/or vancomycin in serum, we were able to demonstrate the penetration of both agents through an artificial staphylococcal biofilm to the extent that the MIC and MBC for the test organism was exceeded on both sides of the colonized membrane. The combination of vancomycin and rifampin appeared to facilitate the diffusion of vancomycin across the staphylococcal biofilm by more than 1.5 times that attained with vancomycin alone. The converse, however, was not observed, because the combination of both agents reduced the diffusion of rifampin by more than half. The reason for this is unclear, but it might include competition for cellular binding sites, which increases the availability of free drug (as in the case of vancomycin), or the creation or exposure of additional binding sites, which effectively limits the amount of unbound drug.

Using our system, we found that exposure of a staphylococcal biofilm to effective concentrations of rifampin alone failed to sterilize the membrane and promoted high-level resistance of surviving organisms. This is in contrast to the findings of Obst et al. (13), who found that rifampin (and its analogs) effectively sterilized an artificial *S. epidermidis* biofilm. In their report, the time required for biofilm sterilization varied according to the test medium. However, when the biofilm was treated with rifampin in peptone water, occasional foci of resistant survivors occurred at an approximate frequency of $1/10^7$ organisms in the biofilm. They also showed a 2.5-fold increase in the rifampin MBC for their test strain of *S. epidermidis* (0.006 to 0.15 μg/ml) when they tested stationary-phase rather than logarithmic-phase growth. This modest increase does not compare, however, to the $>5 \times 10^5$ increase in the MBC of rifampin for our test organism observed in the present study. The report of Widmer et al. (20) also demonstrated that rifampin is extremely effective against *S. epidermidis* in both in vivo (tissue cages) and in vitro (glass slides) models of foreign body infections and at concentrations far less than the 20-μg/ml concentration used in the present study. Our results, however, are in agreement with the findings of Ein et al. (5), who observed a rapid emergence of rifampin resistance among strains of methicillin-resistant *S. epidermidis* exposed to inhibitory levels of this agent alone.

Unlike rifampin, diffusion experiments with only vanco-

mycin did not promote resistance among surviving organisms but, similarly, did not sterilize the biofilm, even though concentrations more than four to five times the MBC for the test strain were achieved on either side of the membrane. Farber et al. (7) found that extracts of exopolysaccharide from slime-positive strains of *S. epidermidis* antagonize the antimicrobial activity of vancomycin (but not rifampin) in a dose-dependent fashion. This could explain the increased level of resistance of organisms embedded in a biofilm to vancomycin. Obst et al. (13) also reported incomplete sterilization of *S. epidermidis* biofilms when vancomycin was suspended in peptone water or spent or buffered peritoneal dialysis fluid, demonstrating the influence of the microenvironment. The combination of rifampin and vancomycin prevented the development of rifampin resistance among surviving organisms, which confirms the observations of Ein et al. (5). The failure of vancomycin plus rifampin to sterilize the biofilm is not surprising and reflects the lack of synergistic activity of the combination against the test organism. In general, the synergistic effect of vancomycin plus rifampin is highly strain dependent for *S. epidermidis* (5, 17). Interestingly, the vancomycin MBC for surviving organisms increased fourfold in the presence of rifampin. As far as we are aware, this phenomenon has not been reported previously and should be examined further.

Using an in vitro model, we found that a staphylococcal biofilm is not impervious to penetration by effective concentrations of vancomycin, rifampin, or both, discounting the theory that implant infections caused by *S. epidermidis* respond poorly to appropriate therapy because of the physical barrier of the biofilm layer. The model described here also cautions against the use of rifampin alone for the treatment of implant infections caused by staphylococci by demonstrating the development of high-level resistance. Although we cannot decisively identify the resistance mechanism(s) of surface-bound CONS, it seems more likely to be a function of the microenvironment or altered bacterial metabolism than barrier protection. This model should prove useful for further evaluations of antimicrobial perfusion through bacterial biofilms.

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